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(54) Title: IDENTIFICATION OF BASES IN NUCLEIC ACID SEQUENCES

(57) **Abstract**

A method of determining whether or not a particular base is present at a specified position in a nucleic acid sequence. The method comprises the steps of (a) providing a single stranded sample of the sequence to be investigated (the "sample sequence") in immobilised form on a solid support, (b) hybridising a primer to said sample sequence such that the base at the 3' end of the primer is hybridised to that base in the sample sequence which is immediately adjacent to the specified position on the 3' side thereof, (c) treating the primer under extension conditions with a labelled moiety capable of providing an extension unit of the primer at a position corresponding to said specified position in the sample sequence if said particular base is present at that position, the extension conditions being such that any extension of the primer does not continue beyond said specified position of the sample sequence otherwise than by further extension with labelled moiety, (d) washing the support to remove unreacted labelled moiety and other reagents, and (e) testing for incorporation of the labelled moiety in the primer.

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IDENTIFICATION OF BASES IN NUCLEIC ACID SEQUENCES

The present invention relates to the identification of bases in nucleic acid sequences. The invention relates more particularly to a method of determining whether or not a particular base is present at a specified position in a nucleic acid sequence. A particular application of the invention is in determining whether or not there is a mutation in a gene.

It is now well established that a number of medical conditions are caused as a result of at least one mutation which has occurred in a gene of the patient. The mutation may, for example, be a deletion which results in the formation of truncated mRNA which in turn results in the formation of a protein having incorrect amino acid residues such that the protein has an incorrect function or no function at all. Alternatively the mutation may be an insert either wholesale (e.g. the introduction of a viral sequence) or on a small scale by evolution or the introduction of single or multiple nucleotides as a result of DNA polymerase misreading. A further example of a mutation is a point mutation which is the result of a base change at a particular site within the genetic code.

Examples of conditions caused by mutations in genes include Gaucher, Cystic Fibrosis, and α and β Thalasseamias. Many of the mutations which cause such diseases are known and it is therefore possible to determine whether a patient is afflicted by, or susceptible to, a particular condition by assaying DNA from the patient to determine whether or not the mutation which causes that condition is present in the DNA. This involves determining whether or not a specific base is present at a particular position in a certain gene sequence.

An example of such an assay technique is disclosed in WO-A-9009455 (Geneco) in which a primer is hybridised to the gene sequence on the 3' side of the base to be investigated, the primer being one which incorporates a detection element or a separation element. An extension reaction is then effected such that, if the specific base is present, the primer is extended to (and only to) that specific base in the sequence being investigated. The base in the extended primer which is

complementary to the specific base in the sequence under investigation incorporates a detection element and/or a separation element such that the extended primer as a whole incorporates both a detection element and a separation element. The presence of the separation element in the extended primer then allows the primer to be denatured from the original sequence and to be exposed to a solid support provided with a moiety having affinity for the separation element. The support is then treated to determine the presence of (or otherwise) of labelled primer immobilised thereon. If (but only if) the label is detected this is confirmation that the specific base was present at the particular position in the sequence under investigation.

The need for affinity separation of the primer is a disadvantage of the technique disclosed in the Geneco Specification because this introduces additional steps into the process and as a consequence limits the potential for automation. The requirement for automation of both single and multiple mutations simultaneously would greatly increase the general applicability of a technique. This is not easily achieved with the abovementioned methodology.

It is therefore an object of the present invention to obviate or mitigate the above mentioned disadvantage.

According to the present invention there is provided a method of determining whether or not a particular base is present at a specified position in a nucleic acid sequence, the method comprising the steps of

(a) providing a single stranded sample of the sequence to be investigated (the "sample sequence") in immobilised form on a solid support,

(b) hybridising a primer to said sample sequence such that the base at the 3' end of the primer is hybridised to that base in the sample sequence which is immediately adjacent to the specified position on the 3' side thereof,

(c) treating the primer under extension conditions with a labelled moiety capable of providing an extension unit of the primer at a position corresponding to said specified position in the sample sequence if said particular base is present at that position, the extension conditions being such that any extension of the primer does not

continue beyond said specified position of the sample sequence otherwise than by further extension with labelled moiety,

(d) washing the support to remove unreacted labelled moiety and other reagents, and

(e) testing for incorporation of the labelled moiety in the primer.

Thus, the method of the invention is used for determining whether or not a particular base is present at a specified position in the sample nucleic acid. If that base is present at that position then a label is incorporated on the primer extension product produced as a result of step (c). The presence of that label, as detected by step (e), confirms that the particular base was present at the position under investigation. The detection may be effected with the primer hybridised to the sample sequence or after denaturation therefrom. In either case, interference from unreacted labelled moiety is avoided by the washing procedure of step (d) which ensures that all such unreacted labelled moiety (and other reagents) are removed prior to the detection step. The method of the invention avoids the need for incorporation of a separation element into the extended primer and a subsequent affinity step for immobilising extended primer on a support. The invention therefore readily lends itself to automation.

The labelled moiety may, for example, be a dideoxynucleotide incorporating the complementary base to that at the specified position in the sample sequence. As such extension of the primer will occur only if the particular base is present at the specified position of the sample sequence and will not continue any further since the dideoxynucleotide terminates the extension reaction. The presence of the labelled species in the primer obviously then confirms the presence of the particular base at the specified position. Instead of using a dideoxynucleotide (or other species which terminate the extension reaction) it is possible to use a labelled nucleotide incorporating a base complementary to that being determined. This might result in extension of the primer beyond the base at the specified position if (on its 5' side) there was an identical base. However, in this case, label would only be incorporated into a primer extension product if the particular base was at the specified position and

the presence of any extra label in the extended primer does not interfere with the process.

The label can be Biotin, allowing the introduction of secondary labels, a fluorophore, enzyme, chemiluminescent tag or similar groups.

The sample nucleic acid may be provided in a number of ways. It is however highly preferred that the sample nucleic acid is covalently linked to the solid support. This may be achieved using the following procedure.

The procedure will start with a sample of nucleic acid ("the original nucleic acid") obtained from a patient. The solid supports will initially be provided with oligonucleotides covalently linked by their 5' ends to the supports. These oligonucleotides will be complementary to a sequence in a strand of the original nucleic acid. By providing that strand in single stranded form, it may be hybridised to the oligonucleotides on the support thereby trapping that strand of the original nucleic acid. After washing to remove non-hybridised material, an extension reaction may be effected so as to extend the oligonucleotides using the trapped original nucleic acid as a template. The original nucleic acid may then be denatured and washed off the supports leaving its immobilised, single stranded copy as the sample nucleic acid on which the method of the invention is effected. It will of course be appreciated that, in this case, the sample nucleic acid is complementary to the original nucleic acid. Therefore in order to determine whether or not there is a particular base at a specified position in the original nucleic acid, it is necessary to determine whether the complement of that base is present at the corresponding position in the sample nucleic acid.

If the amount of the original nucleic acid is relatively low, it is possibly to produce increased amounts of sample nucleic acid for use in the method of the invention using the procedures described in WO-A-93/13220 (Tepnel) and WO-A-95/33073 (Tepnel) thereby producing larger numbers of strands of the sample nucleic acid covalently linked to the support.

Alternatively, it is possible for the solid support initially to be provided with oligonucleotides covalently linked by their 3' ends to the solid support. In this case,

the original nucleic acid may once again be trapped on the oligonucleotides but, in this case, is itself used as the sample nucleic acid.

The solid support preferably comprises particles having a size of 50 to 200 (more preferably 100 to 200) microns. Particularly preferred examples of particles, and methods by which nucleic acids may be covalently linked thereto, are disclosed in WO-A-93/13220.

It is preferred that the particles are provided in a flow-through column into and from which reagents may be readily introduced and exhausted, e.g. as disclosed in WO-A- 93/13220.

In a development of the invention, the method may be used for determining whether or not several different nucleotides are present at specified positions in the sample nucleic acid.

The invention will be further described by way of example only with reference to the accompanying drawings, in which;

Fig.1 illustrates preparation of a sample nucleic acid for analysis in accordance with the method of the invention;

Fig. 2 illustrates a first embodiment of method in accordance with the invention;

Fig. 3 illustrates a second embodiment in accordance with the invention; and

Figs. 4 to 7 illustrate procedures and results of the Examples.

Fig.1 illustrates the production of a solid support system incorporating single stranded nucleic acid (the sample nucleic acid) for use in the method of the invention as described more fully below with reference to Figs. 2 and 3.

The procedure of Fig.1 starts with

(a) a plurality of particulate supports 1 (only one shown) each having a plurality of immobilised oligonucleotides 2 (only one shown) which are covalently linked by their 5' ends to the supports 1, and

(b) single stranded nucleic acid 3 which is to be investigated to determine whether a particular mutation is present therein, the nucleic acid 3 having a region complementary to the immobilised oligonucleotides 2.

In the first step (step (a)) of Fig.1, the strands 3 are hybridised to oligonucleotide 2 using standard reagents and procedures. After washing to remove reagents and non-hybridised materials, the oligonucleotides 2 are extended using a polymerase enzyme and a mixture of dATP, dTTP, dCTP, and dGTP. The extended strands (which are complementary to strands 3) are referenced as 4. The original strands 3 are then denatured and washed off the support leaving the immobilised strands 4 as sample nucleic acid strands.

Assume now that it is necessary to determine whether a strand 3 incorporated, say, the base A at a particular position as a point mutation (as compared. say, to base C in the normal sequence). It is therefore necessary to determine whether the base T or G is incorporated at the corresponding position in strand 4 and the procedure is as outlined below.

Assume now that a strand 4 derived from the 'wild type' strand 3 would incorporate the sequence $^{3'}\underline{T}GCTAG^{5'}$ and that, in a mutant strand 3, the corresponding sequence in strand 4 would be $^{3'}\underline{C}GCTAG^{5'}$. It is therefore necessary to determine whether the 'underlined' base is T or C. This may be effected by the procedure shown in Fig. 2.

As shown in Fig.2, a primer 5 is hybridised to the strand 4 so that the 3' end of the primer is immediately adjacent to that position (marked with a "✚") in the strand 4 at which the base is to be determined. In other words the primer 5 is homologous for the region one base downstream (i.e. on the 3' side of) the possible mutation point "✚".

The supports are then washed to remove reagents and non-hybridised material.

There are now added to the solid support system labelled dATP (*dATP) together with polymerase enzyme. The label is preferably an enzyme label. An extension reaction is the effected followed by washing of the solid support system (leaving the primer 5 hybridised to the strand 4). In the scheme of Fig. 2 it is assumed that the base at the position marked "✚" in the sequence 4 was in fact T so that the primer 5 is extended by labelled A (i.e. *A)

A detection procedure is then effected to determine whether labelled dTTP has been incorporated in the primer 5. For the procedure illustrated in Fig. 2 the result would be positive. If the detection procedure had proved negative, labelled dGTP together with polymerase enzyme are added to the support system and an extension reaction again effected using the immobilised primer. After washing of the system, the detection procedure is repeated to confirm that the labelled dGTP has become incorporated on the primer and therefore that the original strand 3 was a mutant.

The procedure described with reference to Fig. 2 makes it possible to assay for single point mutations in a controlled manner. There is no requirement for gel electrophoresis, blotting or other labour intensive and delicate processes. The copy 4 is, in addition, stable and can be repeatedly assayed.

A development of the procedure is shown in Fig.3 and is used for simultaneously determining the presence (or otherwise) of more than one mutation. In this case, up to four primers are simultaneously hybridised to the strand 4, each for determining whether particular mutations are present at specified positions. The series of primers, may be complementary to regions adjacent to a cluster of point or frame shift mutations. They could also be complementary to insert mutation or mutations.

The procedure of Fig.3 is effected on an immobilised complementary copy of the gene sequence.

For the purpose of illustration, it is assumed that the procedure is to investigate the possible presence of up to four point mutations each of which is at the 5' end of one of the sequences 10-13 specifically illustrated in Fig. 3.

Again for the purpose of illustration it is assumed that the four mutations may be detected with the combined use of dATP, dTTP, dCTP and dGTP.

In the first step (step (a)) of the procedure, primers 14-17 are hybridised to each sequence of interest, each primer being homologous for the region one base downstream (i.e. on the 3' side of) the possible point mutations.

The supports are then washed to remove non-hybridised primers and other reagents.

Labelled dATP (i.e. *dATP) is now added to the supports together with polymerase enzyme under extension conditions such that, if one of the mutations is present, the corresponding primer will be extended by the *dATP. The supports are then washed and the procedure repeated sequentially with *dCTP, *dGTP and & *dTTP. An important point to note is that the labels of each of the nucleotides are distinguishable from each other.

At the end of this procedure, a detection procedure is effected on the supports to determine which nucleotides have been incorporated. Since the nucleotides were differently labelled, it is possible to determine which nucleotides. if any, have been incorporated. The different labels may for example be fluorophores which adsorb at different wavelengths.

If all four labelled nucleotides have been incorporated (as shown in Fig. 3) then this demonstrates that four point mutations are present. If less than four mutations are present then it is possible to determine which of the four labelled nucleotides have been incorporated because of their different labellings. It is therefore possible to say which of the mutations are present.

Fig. 3 represents an idealised case in that the four mutations each correspond to a different nucleotide. If two or more of the mutations were the same nucleotide then it would be possible to determine from the strength of the detection signal obtained for any one nucleotide how many mutations corresponding to that nucleotide were present in the sequence.

Example 1

The experiment describes the addition of a biotinylated nucleotide to a probe (DOL024) hybridised to an extended copy of a captured 257bp PCR fragment, 257O. Capture and extension was performed on solid support carrying the 24mer oligonucleotide DOL006. The fragment was captured on this support and an extension copy of this was made by initiating extension of the support-bound oligonucleotide using AmpliTaq polymerase (Perkin Elmer) and a mixture of the four

deoxyribonucleotides. The extension product was used to capture the oligonucleotide DOLO24B (see below).

DOL006-5'AGCGGATAACAATTTCACACAGGA

DOL024B-5'(biotin)- CGCCATTCAGGCTGCGCAACTGTT

The experiment was designed such that the probe was located at the distal (3') end of the solid phase extension product, allowing the hybrid, shown in Fig. 4, to be formed. The sequence of the DOLO24 primer was such that the nucleotide dG should be incorporated as the first 5' base. Biotinylated dideoxyGTP was used to test for successful incorporation, while biotinylated deoxyUTP was used to check the specificity of incorporation.

Procedure

The 257O fragment (500 fmol) was introduced to the support (2mg), which was located in flow-through columns. The mixture was denatured (95°C, 5 minutes) prior to capture (37°C. 10 minutes). Following washing to remove unincorporated fragment, extension was initiated by the introduction of an extension mix containing AmpliTaq DNA polymerase (2.5 U μL^{-1}) and a mixture of the four deoxyribonucleotides (0.2 pmol μL^{-1} each) in 1 x PCR buffer (supplied with the polymerase enzyme). Extension proceeded during an incubation stage (72°C, 5 minutes), after which the captured fragment was removed by "melting" and washing (3 x 95°C, 5 minutes followed by 80 μ L wash) to leave the extended copy attached to the support (see Fig. 4). The extension copy was probed by aspirating 500 fmol of DOLO24 onto the support and capturing (37°C, 5 minutes). Following washing to remove unbound material the nucleotide under investigation was added (25 pmol) together with AmpliTaq DNA polymerase (2.5 U μL^{-1}) in 1 x PCR buffer. Incorporation of the nucleotide was achieved during incubation at 72°C for 5 minutes. Following extensive washing to remove unincorporated label, incorporated material

was detected by binding of a Streptavidin Alkaline Phosphatase conjugate followed by spectrophotometric detection using the AMPAK assay. Controls involved the use of biotinylated capture probe (DOLO24B) at the same levels as DOLO240, above, without nucleotide addition, in order to determine the level of capture of the secondary probe. The results are shown in Fig. 5.

The figure, in the absence of 257O or of extension of captured fragment, little or no signal is generated with DOLO24B. Extension of 257O allows signficant capture of DOLO24B. The incorporation of ddGTP into unlabelled DOLO240 generates signal approximately half of that generated by DOLO24B capture. Addition of the "wrong" labelled oligonucleotide (dUTP) produces a signal which is less than half that generated by ddGTP addition.

The conclusion drawn from the above observation is that the capture (support-bound) oligonucleotide has been extended. using the captured 257O fragment as template, and that a secondary probe can be bound to this. Further. incorporation of the 5' nucleotide into the probe is sequence-specific.

Example 2

This example demonstrates the feasibility of capturing two primers on single stranded nucleic acid.

The 24mer oligonucleotide DOL006 (see below) was covalently immobilised by its 5' end on particulate solid supports.

The 257bp PCR fragment, 257O, was captured on this support and an extension copy of this was made by initiating extension of the support-bound oligonucleotide using AmpliTaq polymerase (Perkin Elmer) and a mixture of the four

deoxyribonucleotides. The extension product was multiply probed using the biotinylated oligonucleotides DOL024B and

DOLO30B (see below).
DOL006-5'AGCGGATAACAATTTCACACAGGA
DOL030B-5'(biotin)- GGCGTAATCATGGTCATAGCTGTT
DOL024B-5'(biotin)- CGCCATTCAGGCTGCGCAACTGTT

The oligonucleotides are designed such that the two detection probes are located at either end of the solid phase extension product. allowing the multiply primed hybrid shown in Fig. 6 to be formed.

Procedure

The 257O fragment (500fmol) was introduced to the support (2mg), which was located in flow-through columns. The mixture was denatured (95°C, 5 minutes) prior to capture (37°C, 10 minutes). Following washing to remove unincorporated fragment, extension was initiated by the introduction of an extension mix containing AmpliTaq DNA polymerase (2.5 $U\mu L^{-1}$) and a mixture of the four deoxyribonucleotides (0.2 pmol μL^{-1} each) in 1 x PCR buffer (supplied with the polymerase enzyme). Extension proceeded during an incubation stage (72°C, 5 minutes), after which the captured fragment was removed by "melting" and washing (3 x 95°C, 5 minutes followed by 80 μ L wash) to leave the extended copy attached to the support. The extension copy was probed by aspirating 500 fmol of detection probe onto the support and capturing (37°C, 5 minutes). Following washing to remove unbound material secondary probes, where necessary, were captured in exactly the same way. The columns were then thoroughly washed and the bound probes detected by binding of a Streptavidin Alkaline Phosphatase conjugate followed by spectrophotomeric detection using an AMPAK assay.

The results are shown in Fig. 7 which demonstrates that the signal generated by the addition of both detecton probes, in the absence of extension of the support bound oligonucleotide, is similar to background. The addition of each detection prove to support-bound oligonucleotide which has undergone extension generates significant signal. Addition of both detection probes simultaneously produces a signal which exceeds that generated by addition of the invidivual detection oligonucleotides separately.

The conclusion drawn from the above observation is that the capture (support-bound) oligonucleotide has been extended, using the captured 257O fragment as template, and that the detection oligonucleotides bind to this extension product independently of each other.

CLAIMS

1. A method of determining whether or not a particular base is present at a specified position in a nucleic acid sequence, the method comprising the steps of

(a) providing a single stranded sample of the sequence to be investigated (the "sample sequence") in immobilised form on a solid support.

(b) hybridising a primer to said sample sequence such that the base at the 3' end of the primer is hybridised to that base in the sample sequence which is immediately adjacent to the specified position on the 3' side thereof.

(c) treating the primer under extension conditions with a labelled moiety capable of providing an extension unit of the primer at a position corresponding to said specified position in the sample sequence if said particular base is present at that position, the extension conditions being such that any extension of the primer does not continue beyond said specified position of the sample sequence otherwise than by further extension with labelled moiety,

(d) washing the support to remove unreacted labelled moiety and other reagents, and

(e) testing for incorporation of the labelled moiety in the primer.

2. A method as claimed in claim 1 wherein the labelled moiety is a dideoxynucleotide incorporating the complementary base to that at the specified position in the sample sequence.

3. A method as claimed in claim 1 wherein the labelled moiety is a labelled nucleotide incorporating a base complementary to that being determined.

4. A method as claimed in any one of claims 1 to 3 wherein the label is biotin.

5. A method as claimed in any one of claims 1 to 4 wherein the sample sequence is covalently linked to the solid support.

6. A method as claimed in claim 5 wherein the covalently linked sample sequence has been obtained by

providing a sample of nucleic acid ("the original nucleic acid")·

providing solid supports having oligonucleotides covalently linked by their 5' ends to the supports, the oligonucleotides being complementary to a sequence in a strand of the original nucleic acid

hybridising said strand of the original nucleic acid to the oligonucleotides

washing to remove non-hybridised material

extending the oligonucleotides using the hybridised original nucleic acid as a template, and

denaturing the original nucleic acid and washing off the support to leave the immobilised sample nucleic acid.

7. A method as claimed in any one of claims 1 to 6 wherein the solid support comprises particles having a size of 50 to 200 microns.

8. A method as claimed in claim 7 wherein the particles are provided in a column into and from which reagents may be readily introduced and exhausted.

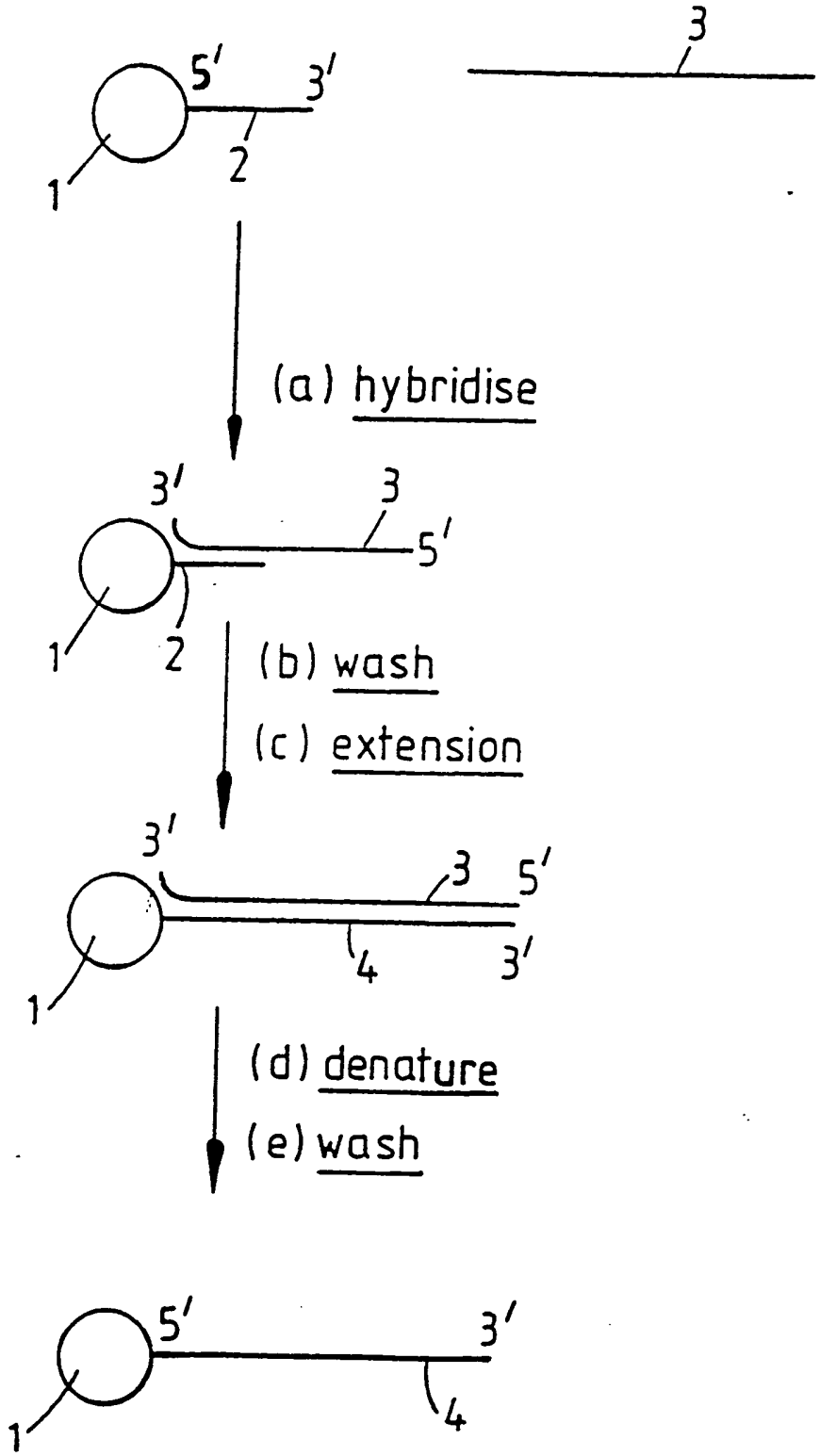
3
5'
3'
2
1
(a) hybridise
3'
3
5'
1
2
(b) wash
(c) extension
3'
3
5'
4
3'
1
(d) denature
(e) wash
5'
3'
4
1

FIG.1

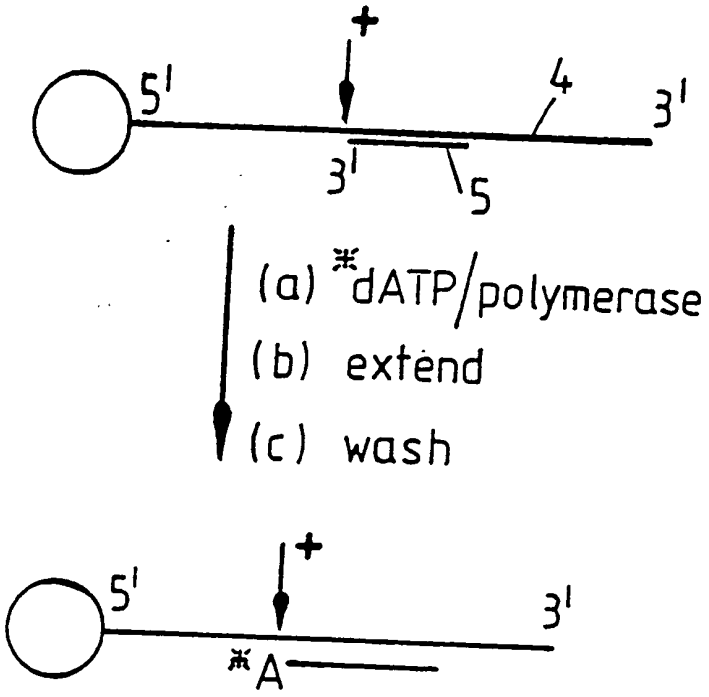

FIG. 2

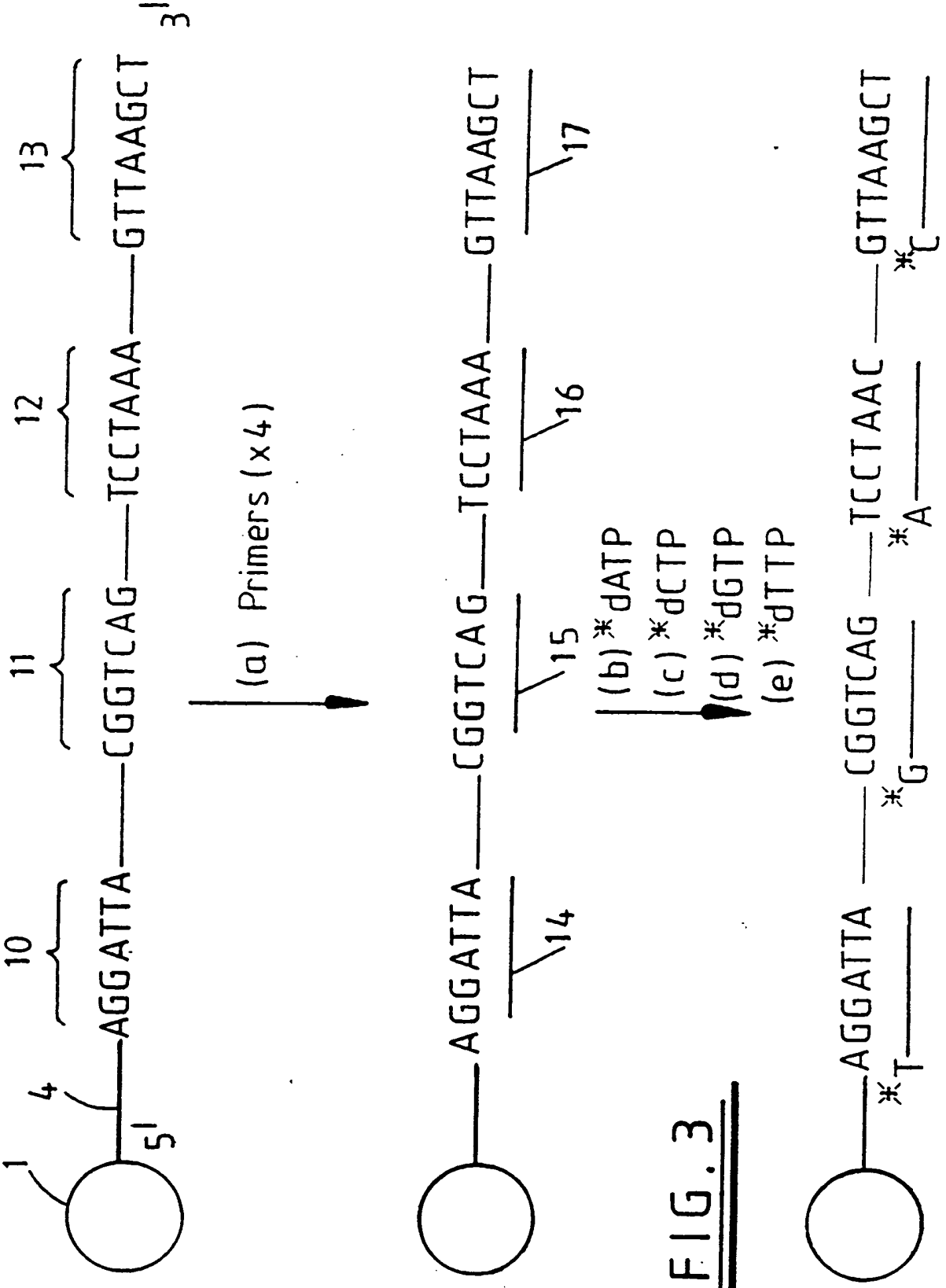
1
4
5'
AGGATTA
10
CGGTCAG
11
TCCTAAA
12
GTTAAGCT
13
3'
(a) Primers (x4)
AGGATTA
14
CGGTCAG
15
TCCTAAA
16
GTTAAGCT
17
(b) *dATP
(c) *dCTP
(d) *dGTP
(e) *dTTP
AGGATTA
*T
CGGTCAG
*G
TCCTAAC
*A
GTTAAGCT
*C

FIG. 3

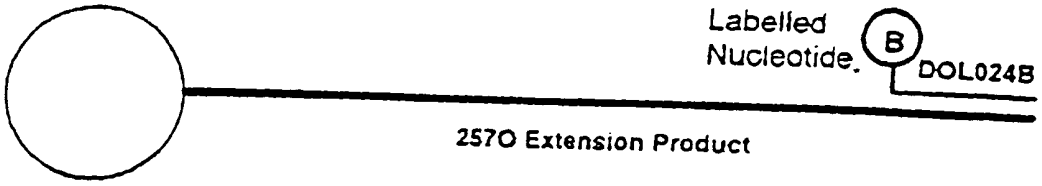
Labelled
Nucleotide
B
DOL024B
2570 Extension Product

FIG. 4

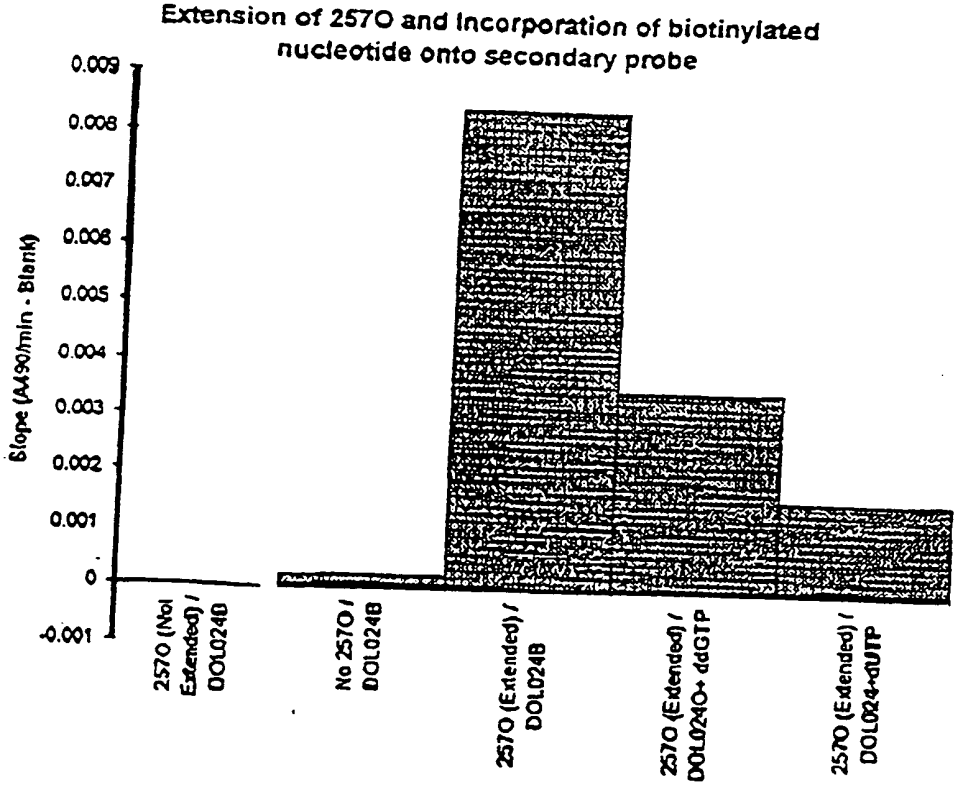
Extension of 2570 and Incorporation of biotinylated nucleotide onto secondary probe
Slope (A490/min - Blank)
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
-0.001
2570 (Not Extended) / DOL024B
No 2570 / DOL024B
2570 (Extended) / DOL024B
2570 (Extended) / DOL024B+ ddGTP
2570 (Extended) / DOL024B+dUTP

FIG. 5

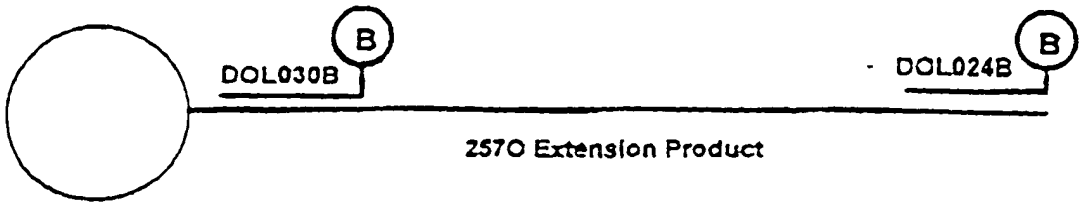
B
DOL030B
B
DOL024B
2570 Extension Product

FIG. 6

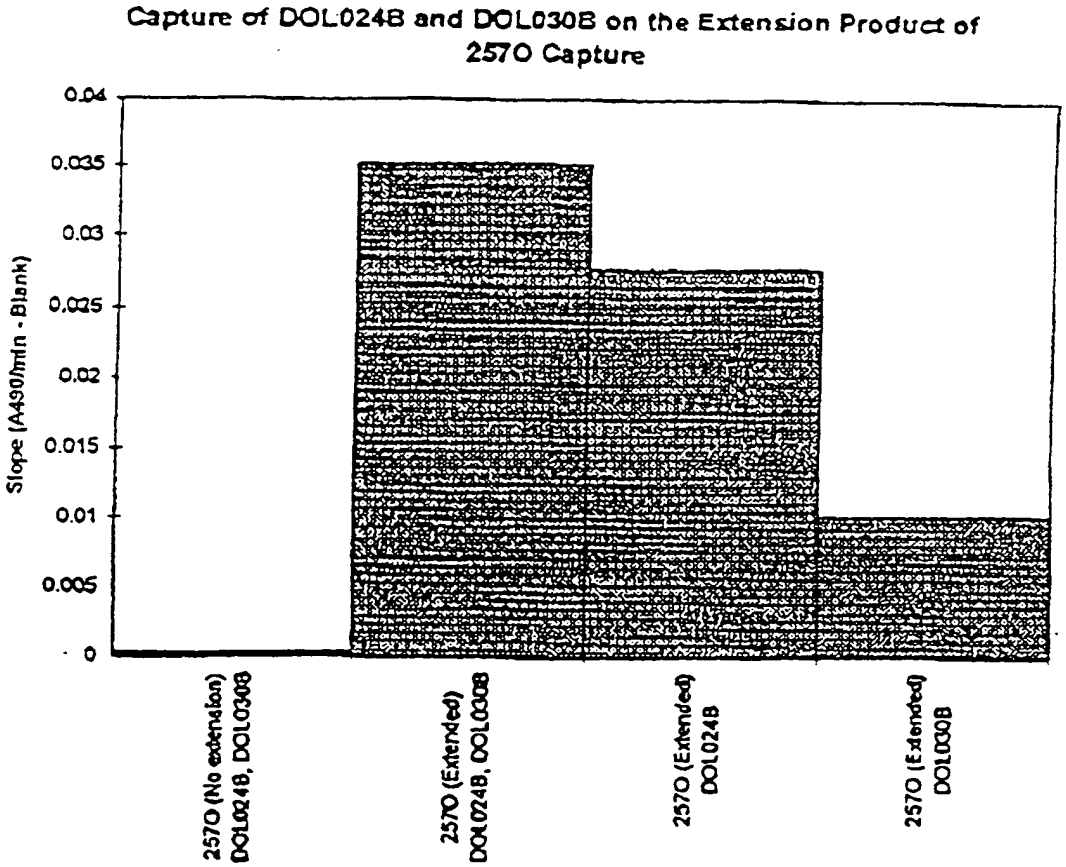
Capture of DOL024B and DOL030B on the Extension Product of 2570 Capture
Slope (A490/min - Blank)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
2570 (No extension) DOL024B, DOL030B
2570 (Extended) DOL024B, DOL030B
2570 (Extended) DOL024B
2570 (Extended) DOL030B

FIG. 7

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB,A,2 283 815 (TEPNEL MEDICAL LTD.) 17 May 1995 see page 4, paragraph 5 - page 6, paragraph 1	1-3,6-8
X	WO,A,91 13075 (ORION YHTYMÄ OY) 5 September 1991 see the whole document	1-3,6-8
X	WO,A,93 13220 (TEPNEL MEDICAL LTD) 8 July 1993 cited in the application see page 14, paragraph 2	1-3,5-8
X	WO,A,92 16657 (E.I. DU PONT DE NEMOURS AND CO.) 1 October 1992 see claims 1-10	1-3,5-8

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1997	14.02.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Osborne, H

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International Application No
PCT/GB 96/02136

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,90 09455 (GENECO PTY LTD) 23 August 1990 see page 29, paragraph 2 - page 31, paragraph 1; figure 1	1-3,5-8
Y	WO,A,93 23562 (CEMU BIOTEKNIK AB) 25 November 1993 see claims 1-4	1-3,5-8

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